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Analysis of Nerve Agent Metabolites from Hair for Long-Term Verification of Nerve Agent Exposure

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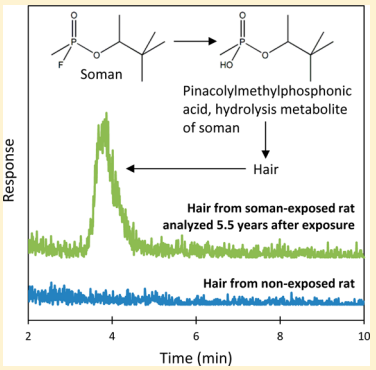
ABSTRACT: Several methods for the bioanalysis of nerve agents or their metabolites have been developed for the verification of nerve agent exposure. However, parent nerve agents and known metabolites are generally rapidly excreted from biological matrixes typically used for analysis (i.e., blood, urine, and tissues), limiting the amount of time after an exposure that verification is feasible. In this study, hair was evaluated as a long-term repository of nerve agent hydrolysis products. Pinacolyl methylphosphonic acid (PMPA; hydrolysis product of soman) and isopropyl methylphosphonic acid (IMPA; hydrolysis product of sarin) were extracted from hair samples with *N*,*N*-dimethylformamide and subsequently analyzed by liquid chromatography−tandem mass spectrometry. Limits of detection for PMPA and IMPA were 0.15 μg/kg and 7.5 μg/kg and linear ranges were 0.3−150 μg/kg and 7.5−750 μg/kg, respectively. To evaluate the applicability of the method to verify nerve agent exposure well after the exposure event, rats were exposed to soman, hair was collected after approximately 30 days, and stored for up to 3.5 years prior to initial analysis. PMPA was positively identified in 100% of the soman-exposed rats (N = 8) and was not detected in any of the saline treated animals (N = 6). The hair was reanalyzed 5.5 years after exposure and PMPA was detected in 6 of the 7 (one of the soman-exposed hair samples was completely consumed in the analysis at 3.5 years) rat hair samples (with no PMPA detected in the saline exposed animals). Although analysis of CWA metabolites from hair via this technique is not appropriate as a universal method to determine exposure (i.e., it takes time for the hair to grow above the surface of the skin and typical analysis times are >24 h), it complements existing methods and could become the preferred method for verification of exposure if 10 or more days have elapsed after a suspected exposure.



Chemical warfare agents (CWAs) were first introduced on the modern battlefield during WWI. Since that point, multiple novel CWAs have been identified, including nerve agents. Nerve agents are of considerable concern due to their extreme toxicity. Sarin, soman, and VX, the most toxic man-made substance known, have dermal LD_{50} values of 24 mg/kg, 5 mg/kg, and 0.14 mg/kg, respectively.[1,2]

When exposed to nerve agents, inhibition of acetylcholinesterase (AChE; Figure 1, reaction A) at the serine active site (compound 1) causes the buildup of the neurotransmitter acetylcholine to produce symptoms including copious secretions, loss of consciousness, convulsions, and apnea. While these symptoms may be obvious at high-doses, symptoms of a low-dose exposure (i.e., pupil constriction, rhinorrhea, and mild breathing difficulties) may not be as apparent.[3,4]

Nerve agent exposure can currently be verified by targeting three classes of compounds of interest: (1) alkyl methylphosphonic acids (AMPAs),[5−12] (2) reactivated nerve agents,[13−17] and (3) nerve agent−protein adducts.[18−26] Direct hydrolysis of nerve agents, as shown in Figure 1, reaction E, produces AMPAs (compound 5). AMPAs, however, can also be formed by the release of the nerve agent residue from the serine active site on the enzyme by hydrolysis (Figure 1, reaction D). Reactivated nerve agents are produced when the enzyme-agent complex (compound 3) is reacted with excess KF (reaction B) to reform the original nerve agent and enzyme. The final class of compounds used to verify exposure is nerve agent−protein adducts. The general reaction between proteins and nerve agents is depicted in Figure 1, reaction A, to form agent−protein adducts (compound 3). While the figure depicts reaction with a serine residue, nerve agents have also been shown to react with tyrosine residues on proteins and peptides.[26]

While several methods have been used to detect exposure to nerve agents,[8,10,27−29] the main concern with these approaches is that the target parent agents and metabolites have short residence times in the body. The analysis of each of the three classes of compounds used to determine nerve agent exposure listed above has drawbacks. Hydrolysis products are only detectable in most biological matrixes for up to 10 days because of further metabolism and excretion.[7,8] Reactivated nerve agents are greatly affected by aging (a process in which the alkyl

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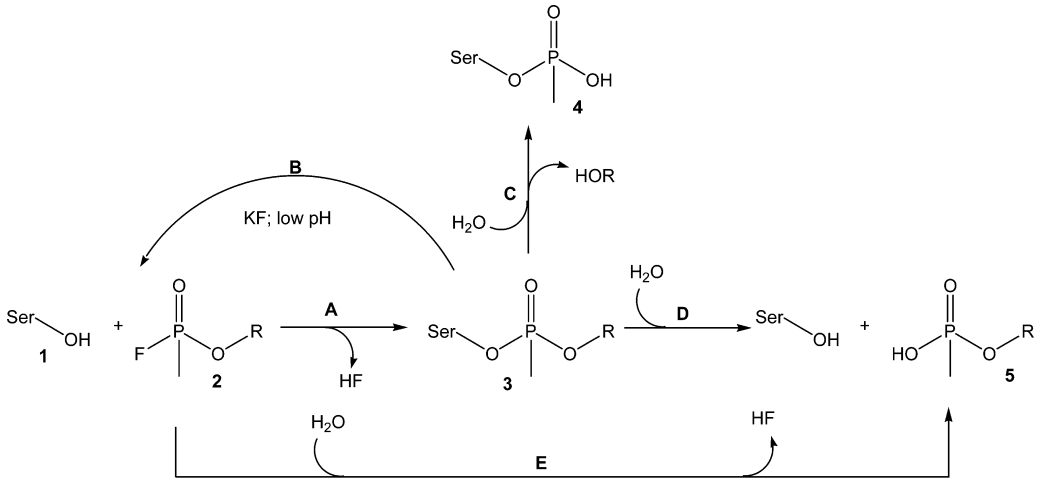

Figure 1. Fate of sarin and soman after binding to serine at the active site of AChE (reaction A). Fluoride reactivation of the AChE enzyme is shown in reaction B. Reaction C depicts aging of the agent−enzyme complex. Reaction D represents cleavage of the agent from the enzyme via hydrolysis. Reaction E describes direct hydrolysis of the parent nerve agent. In the figure, R represents the pinacolyl group of soman or the isopropyl group of sarin.

Table 1. Current Windows of Detection of Nerve Agents

class	analyte	matrix	analysis method	window of detection (experimental)[a]	investigators
AMPAs	IMPA	urine	GC/MS, GC/MS/MS, GC-FPD	2 days	Shih et al.[7] Minami et al.[8]
		blood[b]	LC−MS/MS	1.5 h, single analysis	Noort et al.[10]
		blood[b]	GC/MS	14 h	Shih et al.[7]
		blood[b]	LC−ESI-MS-TOF	180 min	Evans et al.[11]
	PMPA	urine	GC/MS	2 days	Shih et al.[7]
		blood[b]	GC/MS, GC/MS/MS	5 min, single analysis	Fredriksson et al.[9]
reactivated nerve agent	reactivated sarin	blood[b]	GC-NP, GC/MS	5−10 days	Polhuijs et al.[15]
		tissue	GC/MS	5−10 days	Adams et al.[16]
	reactivated soman	blood[b]	GC/MS	5−10 days	Adams et al.[16]
		tissue	GC/MS	5−10 days	Adams et al.[16]
protein adducts	inhibited human butyrylcholinesterase	blood[b]	ESI-MS/MS	1.5 h, single analysis	Fidder et al.[21]
		blood[b]	spectrophotometry (Ellman's assay)	15 min-24 h	Che et al.[22]
	phosphylated tyrosine[d]	blood[b]	SPE-LC−MS/MS	Up to 24 days	Read et al.[39]
		blood[b]	LC−IDMS-MS	48 h	Bao et al.[24]
		blood[b]	immunoassay	15 days	Chen et al.[25]
		blood[b]	LC−MS/MS	45 min−7 days, single analyses[c]	Williams et al.[26]

[a]Some of the window of detection values correspond to individual samples and not a time course of samples evaluated following an exposure. These types of studies are noted as "single analysis" in the table. In cases where several samples were obtained, the longest amount of time that the analyte was detected is listed. Therefore, the window of detection values listed represent the longest experimentally verified time where an analyte was detected following an exposure, and not necessarily a true window of detection. [b]Blood matrix indicates whole blood, plasma, or serum was used for analysis. [c]Multiple agents, each individually analyzed at different points after exposure. [d]In the case of Chen et al., model compounds were used to simulate nerve agent adducts.

side chain is cleaved from the nerve agent-protein adduct), after which the agent cannot be regenerated. Sarin adducts undergo slow aging (a few hours) while soman adducts age within minutes.[15,30,31] However, in either case, the aging process precludes the use of this technique for determination of exposure. The analysis of nerve agent−protein adducts usually requires enzymatic digestion and is thus time and labor intensive.[21] Moreover, while nerve agent−protein adducts may allow for verification while the adducted protein is circulating, the half-lives of the affected proteins are relatively short (e.g., in mammals, the half-life of AChE ranges from 40 h to 2.84 days[32−34] and the half-life of BuChE ranges from 21 h to 5 days[35−38]).

Table 1 lists some methods of retrospective nerve agent analysis methods and their corresponding window of detection (i.e., the maximum amount of time a method or target analyte has been used to verify exposure following an acute toxic agent exposure). With limited windows of detection, the chance for retrospective detection of an exposure is limited if a significant amount of time has passed. In cases where an individual is not exposed to a large enough dose to cause severe symptoms, incorrect diagnosis and treatment could result. Over time, the biomarkers currently used for verification of nerve agent exposure are metabolized and excreted from the body. Therefore, depending on the elapsed time, there may be no method to positively identify a past exposure from typical

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biological matrixes (i.e., blood, plasma, and urine). This issue can be overcome by discovery of an extremely stable biomarker or a matrix that can preserve known metabolites for long periods of time.

Nerve agent metabolites within the bloodstream are transferred to the hair cells formed in the hair follicle when the follicle is nourished by capillary blood vessels. Once the cells are keratinized, the metabolite is tightly bound at the center of the growing hair shaft, protecting it from further metabolism.[40] After the segment of hair shaft containing the agent or metabolite grows above the skin, the hair can be sampled, extracted, and analyzed to verify a past exposure. Hair analysis was first used for the detection of heavy metals, followed by opiates and other drugs of abuse.[41] The analysis of hair has been extensively studied for popular drugs of abuse, such as cocaine, amphetamines, codeine, morphine, and marijuana.[42−48] Researchers have also used hair samples to identify victims of sexual assault.[42,49−53] In addition, hair samples have also been used to study exposure to pesticides and other pollutants.[42,54−60] For example, Tutudaki et al.[61] found that diazinon was present in the hair of rabbits after chronic exposure over a time period of 4 months. Diazinon is an organophosphate pesticide sometimes used as a nerve agent surrogate because of its similar structure, properties, and mechanism of action. The same researchers performed a similar study using rats (45 days), with similar results.[62] Because of its ability to protect metabolites from further metabolism and excretion, hair shows promise as an alternative matrix for retrospective determination, potentially providing a longer window of verification (possibly years) than other methods currently available for nerve agent exposure (Table 1).[42,48]

Because the symptoms from low-dose nerve agent exposure may be initially dismissed and exposure later suspected, or the exposed individual may not have ready access to medical services, a method able to verify nerve agent exposure at extremely long time periods after a suspected exposure would be very useful.[8,15,16,63,64] Therefore, the objective of this work was to develop a method for detection of PMPA and IMPA from the hair of soman and sarin exposed individuals as the first technique to use hair to verify exposure to these toxic agents.

MATERIALS AND METHODS

Materials. All solvents (HPLC grade or higher) and formic acid (≥99%) were obtained from Fisher Scientific (Hanover Park, IL). Pinacolyl methylphosphonic acid, isopropyl methylphosphonic acid, (PMPA, IMPA; each 1000 μg/mL in methanol), D_7-IMPA (1000 μg/mL in methanol; 96% isotope purity), and $^{13}C_6$-PMPA (100 μg/mL in methanol; 95% isotope purity) were obtained from Cerilliant (Round Rock, TX). Aqueous stock solutions (1 μg/mL) were prepared and stored at room temperature. Analytical grade water (18.2 MΩ cm resistivity) was obtained from a Labconco Water Pro PS water purification system.

Hair Samples. Human hair samples used for validation experiments were collected from volunteers under the guidelines approved by the Institutional Review Board of South Dakota State University. Volunteers were allowed to collect and submit their own hair. Gender, race, nationality, health, etc. of the volunteers was not a factor in accepting the hair samples, but any hair that was chemically treated (i.e., permed, dyed, or bleached) was not accepted. If necessary, the hair was cut into short lengths (approximately 1−2 cm) and stored at room temperature until used. If possible, hair from a single volunteer comprised all samples within a single experiment. When more than one volunteer's sample was used within an experiment, the different hair types were never pooled in an individual sample. To remove any external contamination, the hair was initially washed with a 1% sodium dodecyl sulfate solution. The hair was then rinsed three times with deionized water. Finally, the hair was rinsed with methanol, allowed to air-dry overnight, and was stored at room temperature until used.

Rat hair samples for method application were obtained from the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) through animal studies conducted in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health.[65] All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). During the study, all animals were single housed and fed a Teklad Rodent Diet No. 8604, with food and water freely available. Bioserve treats were given once per week for enrichment. Six male Sprague−Dawley rats (Charles River Laboratories, Wilmington, MA) weighing approximately 300 g were subcutaneously (sc; lower right flank at hip level) administered saline, while eight rats were exposed (sc) to 1.2 LD_{50} (132 μg/kg) soman (GD) in the hind leg. No supporting therapy was given after exposure. After 1 week of recovery from the exposure, animals were given isoflurane gas to allow EKG activity monitoring once per week. The animals were euthanized after 28−30 days, and hair samples (2.5−5 g) were collected by shaving the back of each individual animal. Because method development had been initiated with human hair well before the rat hair was received, the entirety of the method validation was completed with human hair.

Sample Preparation. Hair extraction was performed by adding 100 mg of hair into a 2.0 mL centrifuge tube, adding aqueous internal standard (1.5 μg/kg $^{13}C_6$−PMPA and 150 μg/kg D_7-IMPA) and 1.5 mL of *N*,*N*-dimethylformamide (DMF), and capping the tube. Spiked and nonspiked (blank) samples were heated for 4 h on a heat block at 70 °C and then shaken for approximately 24 h at room temperature. The DMF was then transferred to a 4 mL glass vial and evaporated at 80 °C to dryness under nitrogen. Dried samples were reconstituted with 0.1% formic acid in a mixture of water and methanol (40:60 water−methanol; 150 μL), syringe-filtered (Millex-GV, 0.22 μm) into a glass insert (150 μL) contained in a screw-top autosampler vial (2 mL), and analyzed by high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS).

Liquid Chromatography−Tandem Mass Spectrometry. HPLC−MS/MS analysis was performed on a Shimadzu LC system (LC-20AD, Shimadzu Corp., Kyoto, Japan) coupled with a Qtrap 5500 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA). Separation was achieved by reversed-phase (RP) chromatography using a Synergi 4 μ Fusion column (50 mm × 2.00 mm; Phenomenex, Torrance, CA). Mobile phase A consisted of 0.1% formic acid in water, mobile phase B was 0.1% formic acid in methanol, and the injection volume was 10 μL. The prepared hair extracts were separated with gradient elution at a flow rate of 0.2 mL/min as follows: the column was initially equilibrated with 60% B, linearly increased to 100% B at 7 min, maintained at 100% B for 1 min, and then decreased linearly back to 60% B over 0.5 min, where the column was equilibrated for the next sample (approximately 2

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min). Data acquisition and peak integration were performed with Analyst software, version 1.4.1.

Detection of analytes was achieved by electrospray ionization (ESI)-MS-MS, operating in negative ion mode. Nitrogen (20 psi) was used as the curtain gas. The ion source was operated at −4500 V, a temperature of 500 °C, and a pressure of 14 psi for nebulizer (GS1) and heater (GS2) gases. The entrance potential of the collision cell was −10 V. Multiple reaction monitoring (MRM) was used for analysis of the analytes (Table 2). The declustering potential and collision energy were optimized for each MRM transition and are shown in Table 2. Quantification transitions for IMPA and its internal standard utilized different Q3 masses due to an unstable baseline in the D_7-IMPA transition of m/z 144 → 79.

Table 2. Selected MRM Transitions, Optimized Declustering Potentials (DP) and Collision Energies (CE) for Detection of PMPA, IMPA, and Their Internal Standards

compound	Q1Mass (m/z)	Q3Mass (m/z)	time (ms)	DP (V)	CE (V)
PMPA (quantification)	179.1	95.0	40	−151.36	−22.13
PMPA (identification)	179.1	78.8	40	−73.09	−51.75
$^{13}C_6$-PMPA (quantification)	185.0	95.0	40	−89.37	−24.51
$^{13}C_6$-PMPA (identification)	185.0	79.0	40	−43.11	−39.13
IMPA (quantification)	137.0	79.0	40	−95.97	−41.84
IMPA (identification)	137.0	77.0	40	−96.64	−35.13
D_7-IMPA (quantification)	144.3	95.0	40	−111.66	−19.92
D_7-IMPA (identification)	144.3	78.9	40	−105.21	−46.13

Calibration, Quantification, and Limits of Detection. PMPA and IMPA calibration and quality control (QC) standards were prepared in hair as described in Sample Preparation. Two sets of calibration standards for IMPA (0.75, 1.5, 3, 7.5, 15, 30, 75, 150, 300, and 750 μg/kg) and PMPA (0.15, 0.3, 0.75, 1.5, 3, 7.5, 15, 30, 75, and 150 μg/kg) were used to determine the linear range for both analytes. For the calibration curve, a ratio of analyte peak area to internal standard peak area was plotted versus analyte concentration. Nonweighted and weighted ($1/x$ and $1/x^2$) fits were examined with a $1/x^2$ weighted fit producing the best linear description of the calibration data for both PMPA and IMPA as evaluated by the precision and accuracy of the calibration standards. Lower limits of quantification (LLOQ) and upper limits of quantification (ULOQ) were determined via evaluation of calibration standards. Those that had a percent relative standard deviation of <20% (as a measure of precision) as well as an accuracy within ±20% of the nominal concentration back-calculated from the calibration curve, were considered within the linear range. To determine precision and accuracy, three QC concentrations of IMPA (20, 100, and 500 μg/kg) and PMPA (1, 5, and 20 μg/kg) were prepared in hair and analyzed in quintuplicate (N = 5). A new set of high, medium, and low QC standards were analyzed each day for 3 days along with calibration standards (N = 3) for inter- and intra-assay investigations. Precision of the method was considered acceptable if it was <20% and acceptable accuracy was ±20% of the nominal concentration.

Limits of detection (LOD) were determined by analysis of multiple concentrations of PMPA and IMPA. The lowest analyte concentration that consistently produced a signal-to-noise ratio of 3 was defined as the LOD. Noise was determined by observing the baseline noise of the blank hair over the duration of IMPA or PMPA elution.

Selectivity, Recovery, and Stability. Matrix effects were investigated by creating calibration curves for both aqueous and spiked hair (all calibration standards were prepared and analyzed in triplicate) and evaluating the slopes of the resulting curves. Recovery of IMPA and PMPA was determined at low, medium, and high QC concentrations by comparing analyte signals produced from spiked hair and aqueous samples (N = 5). Recovery was calculated as a percentage, relating the peak areas of the spiked hair samples to the peak areas of the aqueous samples.

Short-term autosampler stability was evaluated by placing prepared high IMPA and PMPA QC standards (N = 3) in the LC autosampler. Analysis was performed at approximately 0, 2, 4, 8, 12, and 24 h. Long-term stability of PMPA in hair was evaluated by comparing the results of an initial analysis of the exposed rat hair approximately 3.5 years after collection and a second analysis, approximately 2 years after the first analysis (5.5 years after initial collection).

RESULTS AND DISCUSSION

Analysis of PMPA and IMPA by HPLC−MS/MS. The daughter ion mass spectrum produced by negative ESI-MS-MS analysis for the analytes studied, along with m/z assignments for the fragments, is shown in Figure 2. The m/z ratios of 179 and 137 represent PMPA and IMPA, respectively, ($[M - H]^-$). The MRM transition of m/z 179 → 95 was used for quantification of PMPA, and m/z 179 → 79 was used for identification of PMPA. Corresponding transitions of m/z 185 → 95 (quantification) and m/z 185 → 79 (identification) were observed for the labeled internal standard (i.e., the ^{13}C stable isotope labels are associated with the pinacolyl group of $^{13}C_6$-PMPA). MRM transitions of m/z 137 → 79 (quantification) and m/z 137 → 77 (identification) were observed for IMPA as well as the internal standard transitions of m/z 144 → 95 (quantification) and 144 → m/z 79 (identification). Similar fragments were observed for both analytes as a result of the loss of the side chain from the phosphonyl moiety.

Figure 3 shows representative chromatograms of prepared hair samples. PMPA eluted at approximately 3.8 min and IMPA at approximately 3.5 min. The isotopically labeled internal standards (not shown) coelute with their corresponding nonlabeled analyte. No significant tailing was observed in the IMPA or PMPA spiked hair. The method was very selective, as shown by the lack of coeluting chromatographic peaks for both analytes in the blanks. In fact, no other peaks were observed over 2−10 min for the chromatographic method. A peak corresponding to the void volume of the chromatographic system (not shown) does not interfere with the PMPA or IMPA. No degradation of the aqueous analyte or internal standard solutions used for preparing hair samples was observed over the storage term.

Calibration and Quantification. Calibration curves for PMPA were prepared in hair with an initial concentration range of 0.15−150 μg/kg. Calibrators with a concentration of 0.15 μg/kg did not satisfy the inclusion requirements and

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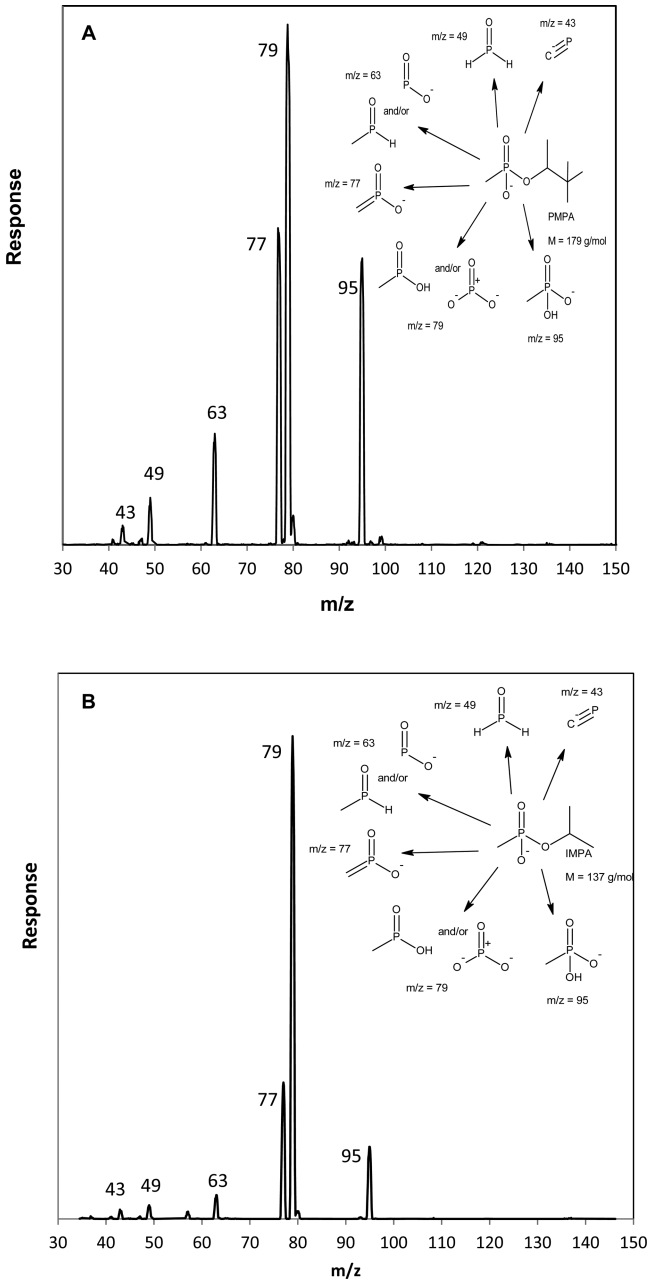

Figure 2. Parent ion fragmentation observed in negative ESI-MS-MS analysis for (A) PMPA and (B) IMPA.

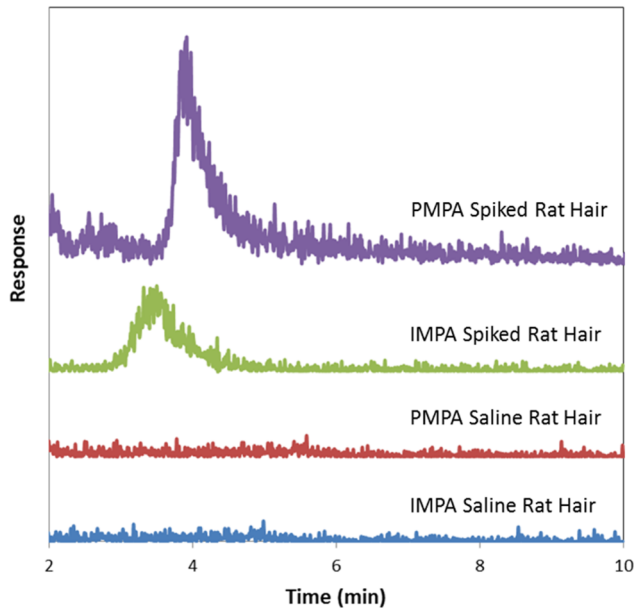

Figure 3. Representative chromatograms of PMPA and IMPA spiked hair (near LODs) samples (upper traces) as compared to saline exposed hair samples (lower traces). Internal standard response is not shown.

were excluded from the calibration range, resulting in a linear range of 0.3−150 μg/kg. Similar calibration curves were prepared for IMPA, with initial concentrations from 0.75 to 750 μg/kg. For IMPA, standard concentrations of 0.75, 1.5, and 3.0 μg/kg did not satisfy the calibration requirements, resulting in a linear range of 7.5−750 μg/kg. While the linear range for PMPA is slightly larger than that of IMPA, both analytes have linear ranges of at least 2 orders of magnitude, which is typical for analysis of biological samples.[66,67] Correlation coefficients (R^2) ranged from 0.9973 to 0.9996 and 0.9955 to 0.9999 for IMPA and PMPA, respectively. The relatively consistent R^2 values show good linearity over the 3 days of QC analysis. For IMPA and PMPA, the slopes of the calibration curves ranged from 0.00473 to 0.00518 and 0.04291 to 0.05491, respectively.

LOD, Accuracy, and Precision. LODs were evaluated from spiked hair samples for PMPA and IMPA and are reported in Table 3, along with the accuracy and precision. Considering the amount of hair and the volume of solvent used to extract the hair, the LODs correspond to 100 pg/mL and 5 ng/mL for PMPA and IMPA, respectively. Therefore, the method presented here achieved comparable or lower detection limits than similar methods for AMPA analysis from biological samples.[63,68−70]

QC standards at low, medium, and high concentrations were evaluated over 3 days of analysis to assess intra- and interassay accuracy and precision. The precision and accuracy were acceptable for both PMPA and IMPA for the concentrations tested but proved much better for IMPA. The intra-assay precision was 11% or less while the interassay precision for IMPA was <9% RSD. IMPA accuracy measurements were within ±10% and ±6% of the nominal QC concentrations for intra- and interassay experiments, respectively. PMPA produced a maximum intra-assay precision of 19% RSD, and an interassay precision of 13% RSD. The accuracy was within ±16% and ±14% of nominal concentrations for intra- and interassay studies, respectively.

Short-Term Stability, Recovery, and Matrix Effects. Short-term stability of AMPAs was evaluated in the autosampler over a 24 h period. Prepared IMPA and PMPA samples at high QC concentrations were stable in the autosampler for at least 24 h. IMPA and PMPA showed accuracy within 12% and 14%, respectively, when compared to the initial analysis of the study (i.e., time zero).

Recovery of IMPA and PMPA were generally consistent across the three concentrations tested (low, medium, and high QCs). IMPA recovery ranged from 41 to 43%, while recovery of PMPA was 35 to 38%. It is observed in the literature that AMPAs analyzed out of other biological matrixes sometimes yield higher recoveries. For example, Hayes et al.[69] demonstrated 95−211% recovery of various AMPAs from saliva samples. However, this is not always the case. Fredriksson et al.[9] showed 58−102% recovery of AMPAs in serum and urine

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Table 3. Accuracy, Precision, LOD, and Recovery of IMPA and PMPA from Spiked Hair Samples

				intra-assay		interassay	
analyte	LOD (μg/kg)	QC concn (μg/kg)	recovery (%)	precision (% RSD)[a]	accuracy (%)[a]	precision (% RSD)[b]	accuracy (%)[b]
IMPA	7.5	20	41	8.7	100 ± 10.0	8.6	100 ± 0.9
		100	43	4.3	100 ± 4.5	4.2	100 ± 4.7
		500	42	2.6	100 ± 4.0	4.3	100 ± 5.3
PMPA	0.15	1	38	11.2	100 ± 13.4	12.3	100 ± 14.0
		5	35	19.4	100 ± 12.5	6.4	100 ± 0.1
		20	37	6.4	100 ± 16.1	6.2	100 ± 2.4

[a]QC method validation (N = 5) for day 3. [b]Mean of three different days of QC method validation (N = 15).

samples, and Katagi et al.[70] only recovered 47−89% of AMPAs from human serum. Comparatively, in analyzing pesticide exposure from hair samples, Tutudaki and co-workers achieved recoveries of 69% from rats and rabbits.[61,62] Margariti and Tsatsakis[56] analyzed dialkyl phosphate metabolites from human hair with 56−108% recovery. While the recoveries achieved in this study are not as high as hoped, they still allowed detection of AMPAs from hair at low limits of detection. Increasing recoveries may be an area of future improvement for the method and may potentially be achieved by modifying the extractant.

Matrix effects were assessed by comparison of spiked aqueous and hair samples. Matrix effects were most prominent for PMPA. The slope of the calibration curve made from the hair samples was 31% of the slope of the calibration curve from the aqueous samples. Although not as prevalent, matrix effects were also observed in the analysis of IMPA. When comparing slopes of the IMPA calibration curves, the slope from the hair samples was 70% of that of the aqueous samples. Although matrix effects were significant for the method presented, the internal standard accurately corrected for these effects, as confirmed by the accuracy of the method. The matrix effects observed may partially account for the low recoveries observed, especially for PMPA.

Verification of Nerve Agent Exposure. The method described here was applied to the analysis of hair samples collected from soman (GD) exposed rats. The exposed hair samples were received from USAMRICD early in method development and required storage until the method was developed and validated. Because the storage of hair samples after an exposure has not been studied, it was unclear as to whether the analyte would be stable in the stored matrix at the chosen storage conditions (−80 °C) or if it would be quantifiable when analyzed by LC−MS/MS. Figure 4 shows chromatograms from the initial analysis (3.5 years after collection) of both saline and GD exposed rats. The figure shows an obvious PMPA peak at approximately 3.9 min in the GD exposed samples, while the saline exposed samples show no signs of PMPA at that retention time. It is very likely that the PMPA seen in the hair is a result of soman in the bloodstream hydrolyzing to PMPA and subsequently being deposited into the hair as the blood nourishes active hair follicles. Although it could be proposed that PMPA may originate from the evaporation of soman, deposition onto the hair of the animal, and subsequent conversion to PMPA during the original exposure, it is highly unlikely. The administration of soman was performed as a subcutaneous injection in the hind leg of the animals by trained personnel in a well-ventilated space. This minimized the chance of evaporation of the soman, and even if a small amount soman did evaporate from the solution inside the syringe, it would likely be swept out of the area before it could deposit on the hair. Another alternative possibility for PMPA detection on the hair is contamination of the hair by urine containing PMPA. It is well-known that soman exposure results in urinary excretion of PMPA (Table 1). While it is possible that if urine came in contact with the hair during the first few days after exposure PMPA could have deposited on the outside of the hair, it is highly unlikely that this type of contamination could occur on the back of the animal (i.e., the hair analyzed was removed from the back). Moreover, the washing procedure described in the Hair Samples section should have removed any external contamination of PMPA prior to analysis by either route of PMPA contamination (i.e., soman vapor contamination or urinary contamination).

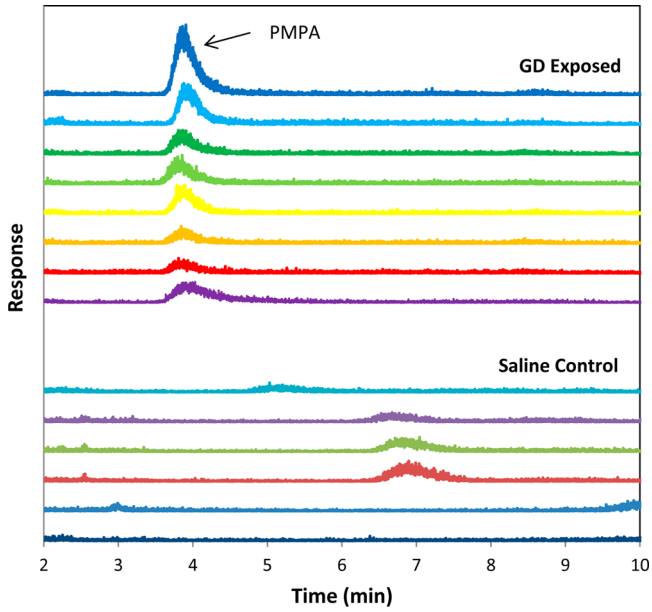

Figure 4. Chromatographic analysis of PMPA from exposed rats collected 1 month after exposure, stored, and then analyzed 3.5 years following exposure. PMPA is clearly evident above the LOD in all the GD exposed rats (calculated concentrations ranged from below the LLOQ up to 11.7 μg/kg) and not present in the hair of saline exposed rats.

A second analysis of this hair was performed approximately 2 years after the initial analysis (5.5 years after collection). This analysis resulted in the saline-exposed rat hair samples showing no signals for PMPA, while the GD exposed samples show a PMPA peak for all samples but one. These results indicate that the analyte can generally be detected in the hair matrix up to 5.5 years with storage at −80 °C. Follow-up studies will be undertaken in the near future to address the stability of the PMPA in hair under a variety of conditions to simulate the typical environmental conditions hair may encounter. Addi-

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tional follow-on studies could include vapor or percutaneous exposure to the agent (i.e., subcutaneous exposure does not approximate a real-life exposure event) and allowing more than 30 days to lapse after exposure but before sample collection.

CONCLUSION

Hair was examined as a matrix for determining past exposure to CWAs. PMPA and IMPA were extracted from hair samples and analyzed by LC−MS/MS. The method produced low limits of detection, good precision and accuracy, and excellent stability. The method showed the ability to detect PMPA from exposed rats when collected 1 month after exposure and analyzed 3.5 years after exposure. Hair also showed the ability to preserve the metabolite under the storage conditions used in this study for over 5 years. Although analysis of CWA metabolites from hair via this technique is not appropriate as a universal method to determine exposure (i.e., it takes time for the hair to grow above the surface of the skin and typical analysis times are >24 h), it complements existing methods and could become the preferred method for verification of exposure if 10 or more days have elapsed after a suspected exposure.

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Notes

The authors declare no competing financial interest.

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